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Thermodynamics of barnase unfolding

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Abstract

The thermodynamics of barnase denaturation has been studied calorimetrically over a broad range of temperature and pH. It is shown that in acidic solutions the heat denaturation of barnase is well approximated by a 2-state transition. The heat denaturation of barnase proceeds with a significant increase of heat capacity, which determines the temperature dependencies of the enthalpy and entropy of its denaturation. The partial specific heat capacity of denatured barnase is very close to that expected for the completely unfolded protein. The specific denaturation enthalpy value extrapolated to 130 °C is also close to the value expected for the full unfolding. Therefore, the calorimetrically determined thermodynamic characteristics of barnase denaturation can be considered as characteristics of its complete unfolding and can be correlated with structural features—the number of hydrogen bonds, extent of van der Waals contacts, and the surface areas of polar and nonpolar groups. Using this information and thermodynamic information on transfer of protein groups into water, the contribution of various factors to the stabilization of the native structure of barnase has been estimated. The main contributors to the stabilization of the native state of barnase appear to be intramolecular hydrogen bonds. The contributions of van der Waals interactions between nonpolar groups and those of hydration effects of these groups are not as large if considered separately, but the combination of these 2 factors, known as hydrophobic interactions, is of the same order of magnitude as the contribution of hydrogen bonding.

Keywords: barnase; hydrogen bonding; hydrophobic interactions; scanning microcalorimetry

Barnase attracts the special interest of researchers engaged in studying protein folding because it is one of the smallest globular proteins (110 amino acid residues; see Kinemage 1) that does not have disulfide crosslinks but is quite stable and unfolds reversibly. The latter circumstance is very important for quantitative analysis of this process by equilibrium thermodynamics, specifying the stability of the native state in energetic terms.

In solutions close to neutral pH, reversible unfolding of barnase by denaturants and temperature appears to be a 2-state transition (Hartley, 1968, 1975; Makarov et al., 1993). Our earlier unpublished calorimetric studies of the heat denaturation of barnase, carried out at the Protein Research Institute in Russia in 1989, showed also that this process is approximated well by a 2-state transition. However, later in 1993 the paper of Makarov et al. (1993) appeared, with calorimetric observations of asymmetry in the melting profile of barnase at low pH values. The asymmetry in melting profile might indicate presence of a stable intermediate in unfolding, perhaps involving folding subdomains. Analysis of the 3-dimensional structure of these proteins does indeed reveal 2 hydrophobic clusters (see Kinemage 1; Serrano et al., 1992). Therefore, the mode of barnase denaturation required detailed investigation. Because there was also some deviation between our estimates of the enthalpy of denaturation and that of Makarov et al. (1993), we felt it necessary to repeat calorimetric measurements on barnase to exclude any ambiguity in the thermodynamics of this protein.

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Results

Figure 1 shows the temperature dependence of the partial molar heat capacity of barnase upon heating in acetate and glycine solutions of various pH. The extensive heat absorption peak is associated with the heat denaturation, which results in a significant increase of the partial heat capacity. With decreasing pH, the thermostability of the protein decreases (Fig. 2), and the enthalpy of denaturation decreases correspondingly.

The process of heat denaturation of barnase is fully reversible in acidic solutions if heating is stopped immediately after completion of the denaturation process. Reversibility decreases significantly in neutral solutions due to aggregation of denatured

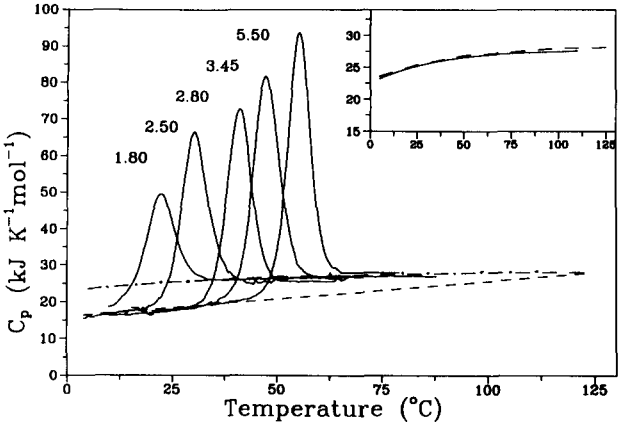

Fig. 1. Temperature dependence of the partial molar heat capacity of barnase in solutions with different pH values, indicated above the curves. The dashed line represents the partial molar heat capacity of native barnase. The dashed-and-dotted line represents the partial molar heat capacity of unfolded barnase. **Inset:** Comparison of the temperature dependencies of the heat capacity of barnase obtained for irreversibly denatured protein by heating it up to 100 °C (solid line), with the heat capacity of the unfolded polypeptide chain C_p^U, calculated according to Privalov and Makhatadze (1990) (dashed line).

molecules. In solutions of pH 7.0 it drops to 30%, judging by the reproducibility of the heat capacity profile. Below pH 1.8 barnase is unfolded at all temperatures. Therefore, in this study we report the results obtained only in the range of pH 1.8–6.0.

Heating of barnase solutions to 100 °C leads to irreversible denaturation of the protein, perhaps because of chemical modification of some groups (Klibanov, 1983).

The partial heat capacity

The partial specific heat capacity of the native barnase is 1.49 J K^{-1} g^{-1} at 25 °C, which is typical for compact globular proteins at this temperature (Privalov et al., 1989). With increasing temperature it increases linearly with a slope of about 0.10 kJ K^{-2} mol^{-1}.

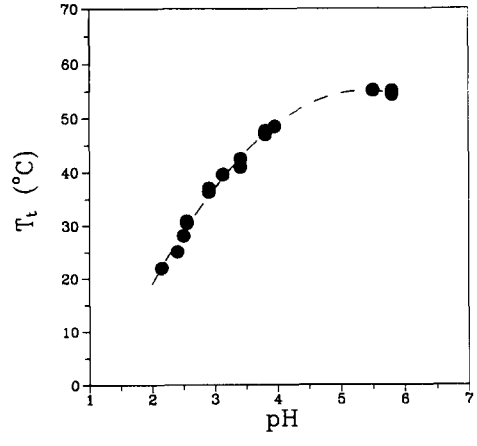

Fig. 2. pH dependence of the denaturation temperature of barnase in 10 mM acetate or glycine buffers.

The heat capacity of denatured barnase is significantly higher than that of native barnase. Its values were measured over a broad temperature range using irreversibly denatured protein, obtained by preheating to 100 °C. The measured values are very close to that of the fully unfolded polypeptide chain of barnase calculated according to Privalov and Makhatadze (1990), using the known heat capacity values of amino acid residues (Makhatadze & Privalov, 1990) and assuming that in the denatured protein all amino acid residues are exposed to water and contribute additively to the heat capacity. The calculated heat capacity function is shown by the dashed line in the inset of Figure 1. The close correspondence between the measured heat capacity of the denatured protein and the calculated one for the unfolded polypeptide chain of barnase is a strong argument that heat-denatured barnase can be regarded as fully unfolded.

In contrast to the heat capacity of the native protein, the heat capacity of the unfolded barnase, as it was expected and found for other proteins, is not a linear function of temperature (Privalov & Makhatadze, 1990; Makhatadze et al., 1993). As a result, the denaturation heat capacity increment clearly depends on temperature (Fig. 1). At 53 °C it amounts to 0.46 J K^{-1} g^{-1} (5.7 kJ K^{-1} mol^{-1}), and one can expect it to drop to 0 at about 130 °C (Privalov & Makhatadze 1990, 1992).

Influence of pH on the thermostability

Decrease of pH leads to a decrease of the area of the heat absorption peak, i.e., the enthalpy of denaturation, and shifts this peak to lower temperatures (Fig. 1). As follows from the pH dependence of the transition temperature, T_t (Fig. 2), barnase is most stable in the region of pH in which it shows a maximal activity of nucleotide hydrolysis (4.5–6.5) (Hartley, 1969; Mossakowska et al., 1989; Pace et al., 1992). In this pH region thermostability of barnase does not depend noticeably on pH, which means that here barnase does not lose or gain protons upon unfolding. The proton uptake upon denaturation, $\Delta_t\nu$, below this pH region can be estimated from the pH dependence of the transition temperature, T_t, using the following equation (Privalov et al., 1969):

$$\Delta_t\nu = -\frac{\Delta H^{cal}(T_t)}{2.303RT_t^2}\frac{dT_t}{d\mathrm{pH}}, \qquad (1)$$

where $\Delta H^{cal}(T_t)$ is the calorimetrically measured enthalpy at the transition temperature, and R is the gas constant. The results suggest that between pH 4 and 2, 3.5 protons are bound on average. This value is close to that obtained previously by Hartley (1969) and by Pace et al. (1992).

The enthalpy of barnase unfolding

The enthalpy values of barnase denaturation obtained from calorimetric experiments are listed in Table 1 and are presented in Figure 3 as a function of transition temperature. In the same figure are presented results obtained from the van't Hoff analysis (Kellis et al., 1989) and calorimetrically by Makarov et al. (1993). The data sets are in good correspondence, but at pH below 3.0 the results of Makarov et al. (1993) deviate systematically. The cause of this deviation is unclear.

It should be noted that because all the calorimetric experiments were carried out in glycine or acetate buffers, the heats

Table 1. *Thermodynamic characteristics of barnase heat denaturation*

pH	T_t (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{cal}/H_{vH}$
1.80	22.0	284	300	0.95
2.15	22.0	307	321	0.96
2.40	25.1	342	353	0.97
2.50[a]	20.0	304	308	0.99
2.55[a]	30.6	354	338	1.05
2.90	36.4	396	402	0.99
2.90[a]	36.5	430	410	1.06
3.12	39.6	401	417	0.96
3.40	42.5	418	435	0.96
3.40[a]	41.0	422	412	0.99
3.80[a]	47.2	442	428	1.03
3.95	48.5	445	466	0.95
5.50	55.1	486	506	0.96
5.80[a]	54.3	525	519	1.01

[a] Data obtained in Russia in 1989.

of ionization of which are very similar to those of protein groups in acidic solutions, these 2 effects balance each other. Therefore, the measured heat effect of protein denaturation actually represents the heat of the conformational transition of protein without protonation effects (Privalov & Potekhin, 1986). This enthalpy change appears as a linearly increasing function of temperature with a slope equal to 5.9 kJ K^{-1} mol^{-1} (Fig. 3). This value is somewhat larger than reported by Makarov et al. (1993), 3.8 kJ K^{-1} mol^{-1}, but is in good correspondence with the denaturation heat capacity increment determined directly from the calorimetrically recorded heat capacity function at $T_t = 50$ °C, the averaged value of which is 5.7 kJ K^{-1} mol^{-1} (Table 2). The difference between the heat capacities of the native and denatured states depends on temperature (Fig. 1). Therefore, the enthalpy of barnase denaturation should not be a linear function of temperature. It only appears as linear (within the accuracy of the enthalpy determination) when it is considered in the experimentally accessible short temperature range. A real temperature dependence of the enthalpy of conformational transition of barnase can be computed using the difference between the heat capacities of the denatured and native states:

$$\Delta_N^D H(T) = \Delta_N^D H(T_t) + \int_{T_t}^{T} \Delta_N^D C_p(T)\, dT, \qquad (2)$$

where

$$\Delta_N^D H(T) = H^D(T) - H^N(T)$$

and

$$\Delta_N^D C_p(T) = C_p^D(T) - C_p^N(T).$$

The $\Delta_N^D H(T)$ function is given in Figure 3 by the solid line.

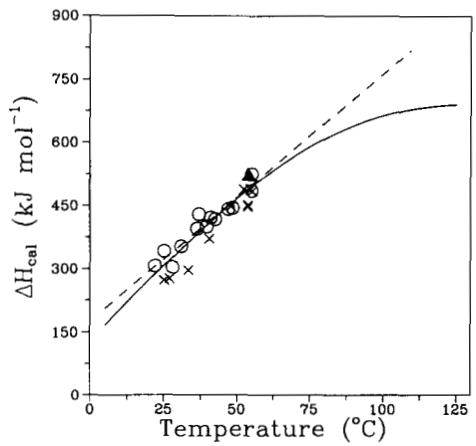

Fig. 3. Temperature dependence of the enthalpy of barnase heat denaturation in solutions with different pH. Circles, calorimetric data obtained in this work; crosses, calorimetric data of Makarov et al. (1993); filled triangles, van't Hoff data of Kellis et al. (1989). The solid line represents the enthalpy of barnase unfolding calculated from Equation 5 using the $\Delta_N^U C_p$ function from Table 2. The dashed line represents the linear fit of enthalpy of barnase heat denaturation versus transition temperature.

Completeness of unfolding

The extrapolation of the enthalpy function to 130 °C gives a value of 56 J g^{-1}. This value is close to the expected one for the complete unfolding of compact globular proteins, 50 ± 5 J g^{-1} (Privalov & Gill, 1988; Makhatadze & Privalov, 1993). Thus, according to the thermodynamic criteria of the enthalpy and heat capacity changes at unfolding, barnase unfolds completely upon heat denaturation.

The mode of denaturational transition

The van't Hoff enthalpies of barnase thermal denaturation determined according to Privalov and Khechinashvili (1974) from the sharpness of the heat absorption peak, with the assumption that this temperature-induced process represents a 2-state transition, are also presented in Table 2. As can be seen, the correspondence between the calorimetric and the van't Hoff enthalpy is very good. The deviation between these enthalpies, the real and effective, does not exceed 3%. Because the van't Hoff enthalpy was determined with the assumption that barnase denaturation represents a 2-state transition, its correspondence to the calorimetric enthalpy means that intermediate states are unstable under all studied conditions, including solutions with very low pH values, down to pH 1.8. Similar conclusions have been made before by Makarov et al. (1993); however, in difference to these authors, we do not see any deformation of the excess heat absorption curve at low pH values, an asymmetry which might indicate that unfolding of barnase is not a simple 2-state transition. Statistical thermodynamic analysis of the shape of the excess heat absorption shows that it is described well by a 2-state transition model. As shown in Figure 4 the observed excess heat capacity profiles are in good correspondence with those expected for a 2-state transition with corresponding enthalpies (see for the statistical thermodynamic analysis of the excess heat capacity Privalov & Potekhin [1986]).

Table 2. *Temperature dependencies of the heat capacity, enthalpy, and entropy for unfolding of barnase*[a]

	Temperature (°C)					
	5	25	50	75	100	125
	Heat capacity (kJ mol^{-1} K^{-1})					
C_p^N	16.5	18.5	21.0[b]	23.5[b]	26.0[b]	28.5[b]
C_p^D	22.6	23.4	24.9	26.4	27.0	28.1
C_p^U	23.6	25.4	26.9	27.4	28.0	28.1
$\Delta_N^U C_p$	7.1	6.9	5.9	3.9	2.0	0.1
	Enthalpy (kJ mol^{-1})					
$\Delta_N^U H_{cal}$	167	307	467	590	664	690
$\Delta_N^U H_{npl}^{hyd}$	−1,260	−952	−590	−250	−74	369
$\Delta_N^U H_{pol}^{hyd}$	−5,954	−6,098	−6,252	−6,391	−6,517	−6,635
$\Delta_N^U H^{int}$	7,381	7,357	7,309	7,231	7,101	6,959
$\Delta_N^U H^{vdW}$	1,037	1,022	999	964	909	830
$\Delta_N^U H^{HB}$	6,344	6,335	6,310	6,267	6,198	6,126
Δh^{HB}	56	56	56	55	55	54
	Entropy (J mol^{-1} K^{-1})					
$\Delta_N^U S^{exp}$	379	866	1,384	1,752	1,959	2,029
$\Delta_N^U S_{npl}^{hyd}$	−4,935	−3,869	−2,693	−1,693	−777	−10
$\Delta_N^U S_{pol}^{hyd}$	−3,281	−3,775	−4,266	−4,672	−5,021	−5,324
$\Delta_N^U S^{cnf}$	8,595	8,510	8,344	8,117	7,758	7,363

[a] C_p^N, the calorimetrically measured and extrapolated values of the partial specific heat capacity of the native protein; C_p^D, the calorimetrically measured partial specific heat capacity of the irreversibly denatured (by heating to 100 °C) protein; C_p^U, the heat capacity of the unfolded polypeptide chain of barnase was calculated from the amino acid composition of the protein as it was suggested earlier:

$$C_p^U = (N-1)\cdot C_p(\text{-CHCONH-}) + \sum_{i=1}^{N} C_p(-R_i),$$

where C_p(-CHCONH-) is the heat capacity of the peptide unit, $C_p(-R_i)$ is the heat capacity contribution of the side-chain of the ith amino acid residue, and N is the number of amino acid residues in the polypeptide chain (Makhatadze & Privalov, 1990; Privalov & Makhatadze, 1990); $\Delta_N^U H_{cal}$, the calorimetrically measured enthalpy of protein unfolding at considered temperature; $\Delta_N^U H_{pol}^{hyd}$ and $\Delta_N^U H_{npl}^{hyd}$ the enthalpies of hydration of polar and nonpolar groups upon protein unfolding, respectively; $\Delta_N^U H^{int}$, the total enthalpy of disruption of internal interactions upon protein unfolding; $\Delta_N^U H^{vdW}$, the enthalpy of van der Waals interactions between nonpolar groups in the protein; $\Delta_N^U H^{HB}$, the total enthalpy of internal hydrogen bonding in the protein; Δh^{HB}, the specific enthalpy of internal hydrogen bonding in the protein; $\Delta_N^U S^{exp}$, the experimentally determined entropy of protein unfolding; $\Delta_N^U S_{pol}^{hyd}$ and $\Delta_N^U S_{npl}^{hyd}$ the entropies of hydration of polar and nonpolar groups upon protein unfolding, respectively; $\Delta_N^U S^{cnf}$, the configurational entropy of unfreezing of the backbone chain and of unpacking of the side-chain groups.

The entropy and Gibbs energy of denaturation

Given that thermal denaturation of barnase represents a simple 2-state transition, at the midpoint of transition, T_t, the Gibbs energy difference between the native (N) and unfolded (U) states is 0:

$$\Delta_N^U G(T_t) = \Delta_N^U H(T_t) - T\cdot\Delta_N^U S(T_t) = 0 \quad (3)$$

and

$$\Delta_N^U S(T_t) = \frac{\Delta_N^U H(T_t)}{T_t}. \quad (4)$$

Thus,

$$\Delta_N^U S(T) = \frac{\Delta_N^U H(T_t)}{T_t} + \int_{T_t}^{T} \Delta_N^U C_p(T)\, d\ln(T) \quad (5)$$

and

$$\Delta_N^U G(T) = \Delta_N^U H(T) - T\cdot\Delta_N^U S(T). \quad (6)$$

The enthalpy and entropy difference between the native and unfolded states of barnase in solutions with pH 5.5 are given in Table 2. These functions do not differ significantly from those for RNase A unfolding (Privalov et al., 1989), but they differ significantly from those of myoglobin unfolding, which has a much larger heat capacity increment than barnase or RNase A.

Discussion

Our calorimetric study of barnase thermal denaturation shows that this process is approximated well by a 2-state transition and its denatured state does not retain thermodynamically significant residual structure. This means that denaturation of barnase can be regarded as a full unfolding process. Thermodynamic functions describing this process can be used to characterize the

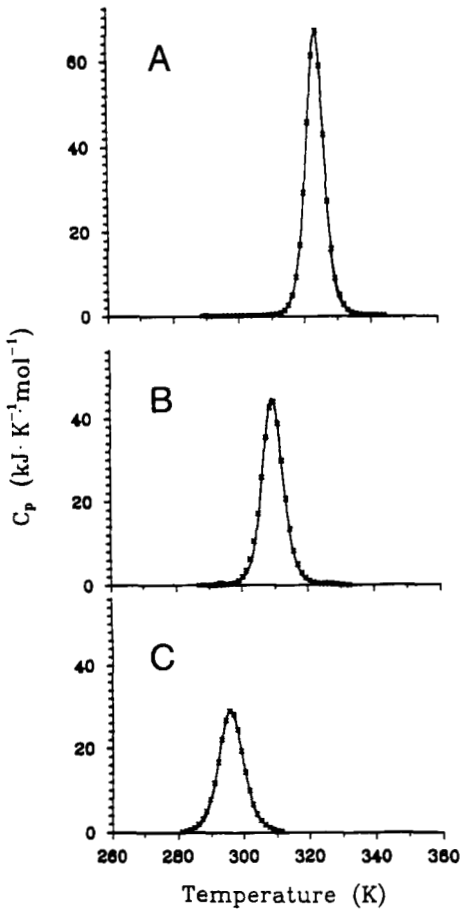

Fig. 4. Excess heat capacity function of barnase in solutions of 10 mM acetate or glycine buffers with different pH. **A:** pH 4.5. **B:** pH 3.0. **C:** pH 1.8. The solid line is the experimentally obtained excess heat capacity function. Crosses, the function calculated assuming that unfolding of barnase is a 2-state transition, with enthalpy change given in Table 1.

energetics of barnase native structure and specify the factors responsible for the folding of the polypeptide chain of barnase to the native conformation.

As has been shown, the heat capacity increment of unfolding of globular proteins is mainly due to the hydration of groups exposed to water upon unfolding (Privalov & Makhatadze, 1990, 1992). Correspondingly, hydration effects should determine the temperature dependencies of the enthalpy and entropy of conformational transition of a globular protein to the unfolded state and contribute essentially to the Gibbs energy difference of the native and unfolded states, which determines the stability of the native structure.

Hydration effects upon barnase unfolding

The hydration effects upon protein unfolding can be calculated, assuming that the groups exposed to water contribute additively to the hydration of protein (Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993):

$$\Delta^{U}_{N}H^{hyd}_{npl}(T) = \sum_{i} \Delta^{U}_{N}ASA_{npl,i} \cdot \Delta\hat{H}^{hyd}_{npl,i}(T) \quad (7)$$

$$\Delta^{U}_{N}H^{hyd}_{pol}(T) = \sum_{k} \Delta^{U}_{N}ASA_{pol,k} \cdot \Delta\hat{H}^{hyd}_{pol,k}(T) \quad (8)$$

$$\Delta^{U}_{N}S^{hyd}_{npl}(T) = \sum_{i} \Delta^{U}_{N}ASA_{npl,i} \cdot \Delta\hat{S}^{hyd}_{npl,i}(T) \quad (9)$$

$$\Delta^{U}_{N}S^{hyd}_{pol}(T) = \sum_{k} \Delta^{U}_{N}ASA_{pol,k} \cdot \Delta\hat{S}^{hyd}_{pol,k}(T). \quad (10)$$

Here $\Delta^{U}_{N}ASA_{npl,i}$ and $\Delta^{U}_{N}ASA_{pol,k}$ are changes of the water-accessible surface areas of the nonpolar and polar groups upon protein unfolding, which are given for barnase in Table 3. $\Delta\hat{H}^{hyd}_{npl,i}(T)$, $\Delta\hat{H}^{hyd}_{pol,k}(T)$, $\Delta\hat{S}^{hyd}_{npl,i}(T)$, and $\Delta\hat{S}^{hyd}_{pol,k}(T)$ are the normalized per square Ångstrom enthalpies and entropies of hydration of polar and nonpolar groups in protein, which were given in Makhatadze and Privalov (1993) and Privalov and Makhatadze (1993).

The calculated hydration enthalpy and entropy of the polar and nonpolar groups of barnase upon unfolding are listed in Table 2. As can be seen, the hydration effects for the polar and nonpolar groups change in opposite direction with increasing temperature.

The intramolecular interactions

Excluding the enthalpy of hydration of polar and nonpolar groups from the calorimetrically determined enthalpy of unfolding, we get the enthalpy of intramolecular interactions in barnase, $\Delta^{U}_{N}H^{int}$ (Table 2). This enthalpy results from the breaking of 2 types of interactions in the protein upon unfolding, van der Waals, and hydrogen bonding:

$$\Delta^{U}_{N}H^{int} = \Delta^{U}_{N}H^{vdW} + \Delta^{U}_{N}H^{HB}. \quad (11)$$

We can estimate the contribution of van der Waals interactions between the tightly packed protein groups by approximating the

Table 3. *Water-accessible surface area change upon unfolding of barnase*

Type of surface	Surface area (ΔASA), Å^2
Aliphatic	5,781
Aromatic	1,666
Polar parts of:	
Arg	324
Asn	229
Asp	252
Cys	0
Gln	102
Glu	23
His	56
Lys	135
Met	0
Ser	122
Thr	76
Trp	67
Tyr	213
-CONH-	2,771

protein interior as an organic crystal, as we did earlier (Makhatadze & Privalov, 1993). The values of the enthalpy of van der Waals interactions in barnase are listed in Table 2. Excluding the enthalpy of van der Waals interactions from the total enthalpy of intramolecular interactions, $\Delta^U_N H^{int}$, we get the enthalpy of hydrogen bonding in the protein, $\Delta^U_N H^{HB}$. There are 113 internal hydrogen bonds in the barnase molecule, calculated from its crystal structure according to Stickle et al (1992). Dividing the total enthalpy of hydrogen bonding by the number of hydrogen bonds in the molecule, we find that 1 hydrogen bond contributes about 55 ± 1 kJ mol^{-1} to the enthalpy of stabilization of barnase. This contribution does not depend significantly on temperature in the considered temperature range.

The entropy of unfolding

The entropy of protein unfolding can be represented as:

$$\Delta^U_N S^{exp} = \Delta^U_N S^{hyd}_{npl} + \Delta^U_N S^{hyd}_{pol} + \Delta^U_N S^{cnf}, \quad (12)$$

where $\Delta^U_N S^{hyd}_{npl}$ and $\Delta^U_N S^{hyd}_{pol}$ are the entropies of hydration of nonpolar and polar groups exposed to water upon unfolding, respectively, and $\Delta^U_N S^{cnf}$ is the configurational entropy of unfreezing of the backbone chain and of unpacking of the side chain groups. The hydration entropies of barnase are listed in Table 2. Knowing the entropies of hydration and experimental entropies of barnase unfolding, one can get the conformational entropy change, $\Delta^U_N S^{cnf}$, using Equation 12. The obtained values of $\Delta^U_N S^{cnf}$ are listed in Table 2. Dividing them by the number of amino acid residues in barnase we get the average contribution of 1 amino acid to the entropy of protein unfolding. It should be noted that the obtained entropy, which equals 8,510 J K^{-1} mol^{-1} at 25 °C, significantly exceeds the value usually used for the configurational entropy of the backbone chain. This is because the configurational entropy that we estimate includes also the entropy of unpacking the side chains and the entropy of disruption of hydrogen bonds, i.e., delocalization of protons, which has never been taken into account.

The Gibbs energy of stabilization of barnase

The components of the Gibbs energy of stabilization of barnase can be calculated from the obtained enthalpy and entropy values of the hydration effects and intramolecular interactions. The result is presented in Figure 5. As one can see, the main positive contributions to the Gibbs energy comes from disruption of internal hydrogen bonds and from the disruption of van der Waals interactions. The hydration of nonpolar groups also stabilizes the structure of barnase, but to a lesser extent. These 3 factors are opposed by the negative effects of hydration of polar groups and increase of configurational entropy.

Because disruption of intramolecular hydrogen bonds is accompanied by immediate hydration of the released polar groups, it is meaningful to group these 2 effects together and consider the overall contribution of hydrogen bonds to the stabilization of the native protein structure, $\Delta G^{HB}_{hyd} = \Delta G^{HB} + \Delta G^{hyd}_{pol}$, as it is usually considered. The situation is the same with van der Waals interactions; whenever they are disrupted upon protein unfolding water hydrates exposed nonpolar groups. This combined effect of disruption of contact between nonpolar groups and hydration of these groups is usually called the hydrophobic effect, $\Delta G^{hph} = \Delta G^{vdW} + \Delta G^{hyd}_{npl}$. In this representation the factors stabilizing the native state of barnase are shown in Figure 6. We see that the contributions of hydrogen bonding and hydrophobic effects to the stabilization of the native state of barnase are of the same order. The absolute values of the hydrophobic effect amounts to 1,200 kJ mol^{-1} or 164 kJ mol^{-1} Å^{-2} at 25 °C. This value is very close to that of 190 ± 60 kJ mol^{-1} Å^{-2} obtained from the study of the effect of hydrophobic mutations on the stability of barnase (Kellis et al., 1988, 1989; Serrano et al., 1992). The total contribution of hydrogen bonding to the Gibbs energy of stabilization of barnase is about 1,350 kJ mol^{-1}. This yields an average contribution of 12 kJ mol^{-1} for 1 hydrogen bond in the protein at 25 °C. Mutational studies give 3-8.5 kJ mol^{-1} for the Gibbs energy value of a hydrogen bond between uncharged groups (Serrano et al., 1992). However,

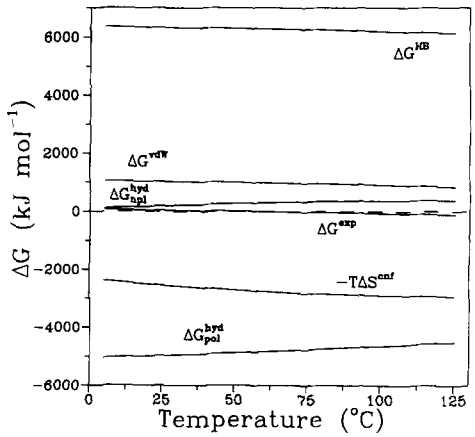

Fig. 5. Relative contribution of various forces to the stability of barnase. Gibbs energy of van der Waals interactions, ΔG^{vdW}; Gibbs energy of internal hydrogen bonding, ΔG^{HB}; Gibbs energy of hydration of nonpolar, ΔG^{hyd}_{npl}, and polar groups, ΔG^{hyd}_{pol}; energy of dissipative forces of thermal motion, $-T\Delta S^{cnf}$; total Gibbs energy of barnase unfolding, ΔG^{exp}, determined from calorimetric studies of its denaturation.

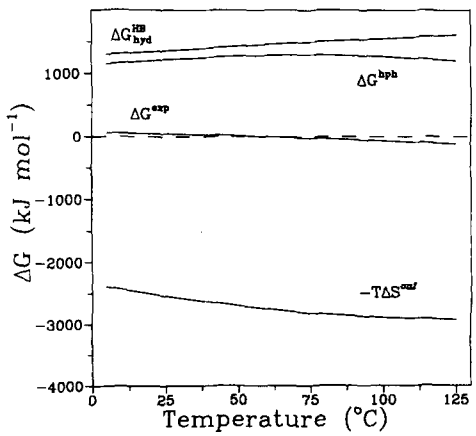

Fig. 6. Relative contribution of various forces to the stability of barnase. Gibbs energy of hydrogen bonding upon unfolding of protein, ΔG^{HB}_{hyd}; Gibbs energy of hydrophobic interactions, ΔG^{hph}; energy of dissipative forces of thermal motion, $-T\Delta S^{cnf}$; total Gibbs energy of barnase unfolding, ΔG^{exp}, determined from calorimetric studies of its denaturation.

these values also include the negative effect of deformation of protein structure upon substitution of 1 amino acid by another with different volume and structure. Therefore, one perhaps should consider as an energy value of a hydrogen bond the upper limit of estimates from mutation studies, i.e., 8.5 kJ mol^{-1}, which is not far from the 12 kJ mol^{-1} we get for the average value of hydrogen bonding. It should be noted that our estimate also includes the bonds between charged groups, which are supposed to be stronger (Serrano et al., 1992) than bonds between uncharged groups.

Experimental

Wild-type barnase was obtained from *Escherichia coli* (XLI-Blue) containing the overexpression plasmid PMT 1002 (Hartley, 1988). After the purification procedure described by Hartley and Rogerson (1972), the purity of the enzyme was about 98% estimated by PAGE under native and denatured conditions (Laemmli, 1970; Spiker, 1980).

The concentration of protein in the solution was determined spectrophotometrically at 280 nm using the extinction coefficient $E_{280nm}^{1cm,1\%} = 22.1$, measured in our laboratory from the nitrogen content using the method described by Jaenicke (1974). The same coefficient has been obtained before by Loewenthal et al. (1991), who have calculated it from the amino acid composition of barnase using the method of Gill and von Hippel (1989). This value is close to the extinction coefficient of 21.0 reported by Hartley (1975). Correction for light scattering was applied according to Winder and Gent (1971).

Calorimetric measurements were performed on a DASM-4 scanning microcalorimeter and a scanning microcalorimeter built at The Johns Hopkins University, at a heating rate of 1 deg/min, using solutions with protein concentration from 0.8 to 1.2 mg/mL.

The partial specific heat capacity of the protein was determined as described elsewhere (Privalov & Potekhin, 1986), assuming that the molecular mass of barnase is 12,382 Da and the partial specific volume is 0.734 $cm^3\ g^{-1}$. The latter was computed from the amino acid composition of barnase according to Makhatadze et al. (1990) and confirmed by direct density measurements using the vibration densitometer DMA-60/602 (Anton Paar).

Thermodynamic analysis of the excess heat capacity function obtained in the calorimetric experiments was performed by the procedure of Filimonov et al. (1982), a modification of a method described by Freire and Biltonen (1978).

The surface area of the native barnase was calculated from the 3-dimensional structure reported by Baudet and Janin (1991) using atomic coordinates of barnase (1rnb) obtained from the Protein Data Bank (Bernstein et al., 1977). Details on the procedure of the surface area calculations were reported elsewhere (Makhatadze et al., 1993). The areas of different types of surfaces in barnase that are exposed to solvent upon unfolding are presented in Table 3.

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